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Enzymatic U(VI) reduction by *Desulfosporosinus* species

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Summary. Here we tested U(VI) reduction by a *Desulfosporosinus* species (sp.) isolate and type strain (DSM 765) in cell suspensions (pH 7) containing 1 mM U(VI) and lactate, under an atmosphere containing N_2-CO_2-H_2 (90 : 5 : 5). Although neither *Desulfosporosinus* species (spp.) reduced U(VI) in cell suspensions with 0.25% Na-bicarbonate or 0.85% NaCl, U(VI) was reduced in these solutions by a control strain, *Desulfovibrio desulfuricans* (ATCC 642). However, both *Desulfosporosinus* strains reduced U(VI) in cell suspensions depleted in bicarbonate and NaCl. No U(VI) reduction was observed without lactate and H_2 electron donors or with heat-killed cells, indicating enzymatic U(VI) reduction. Uranium(VI) reduction by both strains was inhibited when 1 mM $CuCl_2$ was added to the cell suspensions. Because the *Desulfosporosinus* DSM 765 does not contain cytochrome c_3 used by *Desulfovibrio* spp. to reduce U(VI), *Desulfosporosinus* species reduce uranium *via* a different enzymatic pathway.

Introduction

Reduction of U(VI) to U(IV), which immobilizes uranium as the insoluble U(IV) mineral uraninite (UO_2), is a critical transformation in the uranium cycle near the Earth's surface [1]. It has been suggested that sulfate-reducing bacteria (SRB) enzymatically catalyze U(VI) reduction in natural [2–4] and in uranium-contaminated groundwater [5, 6]. Despite the importance of this reaction and the apparent wide phylogenetic diversity of SRB, only six *Desulfovibrio* spp. [4, 7] and one species of *Desulfotomaculum reducens* [8] have been shown in the laboratory to enzymatically reduce U(VI).

When field-collected uranium-contaminated sediments and water from a uranium mine were incubated under anaerobic conditions with organic substrates to stimulate the growth of indigenous anaerobic microorganisms, uranium was almost entirely removed from solution and precipitated as UO_2 [9, 10]. Microbial cells that precipitated UO_2 were also associated with metal sulfides containing Fe, Cu, and Zn. Thus, these cells were inferred to be sulfate-reducing bacteria (SRB). Analysis of 16S rRNA-based clone libraries constructed from the incubated sediment revealed that 42 out of 94 clones were closely related to spore-forming Gram-positive SRB, *Desulfosporosinus* spp. (formerly *Desulfotomaculum*). No organisms related to the other genera of SRB were found in the clone libraries [9, 10]. Based on the correlation between U(VI) reduction, sulfate reduction, and the presence of *Desulfosporosinus* spp., we proposed that *Desulfosporosinus* spp. can enzymatically reduce U(VI).

Materials and methods

Sampling

In July of 2000 sediment was collected from open pit #3 at the Midnite mine, which is an inactive open-pit uranium mine and located approximately 50 miles northwest of Spokane in the state of Washington, USA. The sediment was collected from 50 cm below surface mud, near the water edge. The pit sediment was transferred into an anaerobic jar (Difco Laboratories, Detroit, MI) with a GasPack plus ($H_2 + CO_2$) (BBL, Cockeysville, MD) immediately after collection. The sediment sample was returned to the laboratory within 2 days, kept cold on ice during shipment.

Enrichment and isolation of sulfate-reducing bacteria

For enrichment, 1 g of the pit sediment was inoculated into a serum bottle (100 ml vol) with 50 ml of an autoclaved medium that contained the following (L^{-1}): K_2HPO_4, 0.5 g; NH_4Cl, 1.0 g; Na_2SO_4, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; Na-lactate, 2.0 g; yeast extract, 1.0 g; resazurin, 1.0 mg; $FeSO_4 \cdot 7H_2O$, 0.5 g; Na-thioglycolate, 0.1 g; ascorbic acid, 0.1 g (a Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture medium 63) in an anaerobic chamber (Coy Laboratory, Grass Lake, MI) with an anaerobic gas mixture (AGM) containing N_2-CO_2-H_2 (90 : 5 : 5). The serum bottle was closed with a rubber stopper and an aluminum cover. The culture was incubated at room temperature. The enriched culture was streaked on the medium with 2% agar in the anaerobic chamber filled with the AGM. After 5 transfers, black colonies were enriched and stored separately in the DSMZ medium 63 for further characterization.

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Phylogenetic analysis of isolates

The aliquots (0.1 mL) of the enriched cultures with the black colonies were inoculated in the DSMZ medium 63 (10 mL) depleted with $FeSO_4 \cdot 7H_2O$ to avoid FeS formation because FeS formed during enrichment was found to inhibit PCR reactions after DNA extraction. The enrichment cultures were harvested at their lag phases by centrifugation. The pellets were washed twice with 2 × buffer A (200 mM Tris [pH 8], 200 mM NaCl, 10 mM $CaCl_2$, 50 mM EDTA, 2 mM Na-citrate). The cells were suspended in TE buffer (19 mM Tris [pH 8], 1 mM EDTA), spun down and then resuspended in 100 mL of deionized water. The cells were reciprocated on a Mini Beadbeater (Biospec Products, Inc., Bartlesville, Okla.) at low speed for 2 min. After bead beating, cells were heated at 95 °C for 20 min and then centrifuged at 10 000 g for 20 min. The supernatants containing nucleic acids were purified with ChromaSpin+TE1000 column (Clonetech, Palo Alto, CA).

The 16S rRNA genes of the isolates were amplified by PCR in mixtures containing approximately 40 ng of purified DNA per mL, 1 × PCR buffer (Perkin Elmer, Norwalk, Conn.), 200 μM of each of the four deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, 350 mM (each) forward and reverse primers, and 0.025 U of AmpliTaq Gold (Perkin Elmer) per μL. In reactions, the reverse primer was universal 1492R (5′-GGTTACCTTGTTACGACTT-3′) [11], and the forward primer was *Bacteria*-specific 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) [11]. A Gene Amp 2400 (Perkin Elmer) was used to incubate reactions through an initial denaturation at 94 °C for 12 min, followed by 30 cycles of 94 °C for 1 min, 45 °C for 45 s, and 72 °C for 1.5 min, and completed with an extension period of 20 min at 72 °C. The product was purified using QIAquick PCR purification columns (Qiagen, Valencia, CA).

The purified PCR product was quantified by ethidium bromide-UV detection on a 1% agarose gel. The purified PCR product was sequenced using the Prism Big Dye terminator sequencing kit (Applied Biosystems, Foster City, CA) with 50 to 100 ng of template DNA, according to the manufacturer's instructions. The 16S rDNA sequences of the isolates were obtained using a universal forward primer 533F (5′-GTGCCAGCMGCCGCGGTAA-3′) [9] in addition to the primers 27F and 1492R in sequencing reactions. DNA sequences were determined on an automated sequencer (ABI 377XL) at the University of Wisconsin Biotechnology Center. The sequences obtained by the 3 primers were compiled using SeqEd (Applied Biosystems). The compiled sequence was aligned using the GDE (Genetic Data Environment) multiple sequence editor against close relatives in ARB (a software environment for sequence data) [12]. Unambiguously alignable positions of 16S rDNA sequences were reduced by the lane mask [11]. Evolutionary analysis of aligned sequences was performed by distance methods, parsimony, and maximum likelihood using PAUP [13].

Uranium(VI) reduction experiments

To test U(VI) reduction by *Desulfosporosinus* spp., the stock cultures of the isolate and a type strain (*D. orientis* DSM 765), referred to as strains P3 and DSM 765 respectively, were enriched in the modified DSMZ 63 medium with 0.05 g/L $FeSO_4 \cdot 7H_2O$ (instead of 0.5 g/L) and 0.5 g/L of Na-citrate. This modified medium was used for the first enrichment of cells used for U(VI) reduction experiments to minimize FeS formation. The enriched cultures at the mid-exponential phases were transferred at least twice (10% inoculum) to the other modified DSMZ 63 medium for the U(VI) reduction experiments, in which $FeSO_4 \cdot 7H_2O$ was minimized (0.002 g/L) to support growth for a couple generations. Enriched cells, free from FeS precipitates, were harvested by centrifugation anaerobically. The enriched cells were washed twice. Cell suspensions (10 mL) were prepared in solutions (pH 7) containing 1 mM uranyl acetate and Na-lactate. Three types of cell suspensions, (1) with 0.25% $NaHCO_3$ (bicarbonate), (2) with 0.85% NaCl, and (3) without bicarbonate and 0.85% NaCl were used. To test whether U(VI) reduction was catalyzed by cell components rather than electron transfer from electron donors to U(VI) *via* electron-transfer enzymes, the cell suspensions depleted in Na-lactate and H_2 electron donors were incubated under N_2. One millimolar $CuCl_2$, which inhibits the activity of hydrogenase, was added to subsets of the cell suspensions to check for the involvement of hydrogenase in U(VI) reduction. The cell suspensions containing heat-killed cells, which were prepared by heating cells at 80 °C for 1 h in an oven, were incubated to quantify adsorption of uranium onto the cell surfaces. To quantify U(VI) reduction, subsamples were centrifuged at 10 000 g for 3 min, and total uranium concentrations in the supernatants were measured using a liquid scintillation analyzer (Packard 1900CA). To validate the efficacy of experimental procedures, *Desulfovibrio desulfuricans* [American type culture collection (ATCC) #642], which has been described for the U(VI) [3], was also tested in this study. Cell protein contents in the experimental solutions were measured using the Bradford assay (Bio-Rad, Hercules, CA). Bovine serum albumin was used as reference.

Transmission electron microscopy

The cell suspension of strain P3 (10 mL) incubated for 48 h without bicarbonate or 0.85% NaCl was mounted onto a Cu grid with a formvar film in the anaerobic chamber. The grid was transferred directly into a transmission electron microscope (TEM) (Philip CM 200) for characterization. Chemical compositions of materials in the fraction were analyzed by energy dispersive X-ray spectroscopy (EDX). Mineral phases were characterized by selected area electron diffraction (SAED) analysis.

X-ray absorption near-edge spectroscopy

The same cell suspension of strain P3 characterized by TEM was spun down at 10 000 g for 5 min. The pellet was separated from the supernatant, and mounted in airtight sample holders in the anaerobic chamber for synchrotron-based X-ray absorption near-edge spectroscopy (XANES) analyses. Uranium L_3-edge XANES measurements of this sample were performed at the Material Research Collaborative Access Team (MR-CAT) beamline [14] at the Advanced

Photon Source (APS). The data were collected in fluorescence mode. The undulator gap was tapered to 4 mm to achieve less than 15% change in X-ray energy. An Rh mirror was used to remove high-order harmonics. Slits were used to define the incident X-ray profile of 0.7 mm by 1.0 mm. The incident and transmitted ion chambers were filled with nitrogen gas and the fluorescence ion chamber was filled with Kr gas. Linearity tests of the experimental setup [15] indicated less than 0.1% nonlinearity for a 50% attenuation of the incident X-ray intensity. The fluorescence measurements were made in slew scanning mode with 0.1 s integration time resulting in about 2 min per a XANES scan. Five XANES measurements were made on the sample. No time-dependent change was found in the XANES measurements for any of the samples on the 2 min time scale, indicating that the valence state of the uranium in the samples was not affected by the radiation dose on the sample at these time scales.

The normalization was performed using ifeffit [16] and a Cromer-Libermann normalization algorithm. A Y-foil was monitored during the data collection in a manner previously described [17] to align the XANES data to the U(IV) and U(VI) standards, which were taken from UO_2 and UO_3 powder samples, respectively. The standards, which were diluted in SiO_2, were spread on Kapton tape and measured in fluorescence mode. The U(VI) standard used in these experiments may not be considered ideal as it has been shown in the past to contain up to 25% U(VI). However it could be used to give an approximation of U(IV) edge position.

Results

The DSMZ 63 medium was inoculated with the sediment from the uranium mine. After one week, the medium turned black due to the formation of FeS, indicating the growth of SRB. The enriched medium was streaked onto the agar plates. After 5 transfers of the four black colonies were randomly picked and their identities determined by sequencing their 16S rRNA genes. As the partial 16S rDNA sequences of the 4 isolates, amplified with the F533 primer, had more than 98% similarity, one representative was chosen for further analysis. The full sequence of the representative was analyzed for the phylogenetic affiliation. A phylogenetic tree (Fig. 1) shows that the isolate (strain P3) was firmly clustered with *Desulfosporosinus* spp., and clonal sequences (P3IB-3 and -51), obtained from the incubated uranium-contaminated sediment, as described in the introduction. Members of the genus *Desulfosporosinus* are spore-forming Gram-positive SRB. *Desulfotomaculum orientis* has been systematically separated from the other species of *Desulfotomaculum* and renamed *Desulfosporosinus orientis* [18]. Strain P3 was most closely related to *D. orientis* strain DSM 8344 ($>$ 99% similarity), and more distantly related to strain DSM 765 ($\sim$ 98% similarlity).

Uranium(VI) was reduced by strains P3 and DSM 765 under non-growth conditions. The protein concentrations of the cell suspensions were at ~ 0.06 mg/ml (3.5×10^8 cells/ml). When the cell suspensions contained either bicarbonate or 0.85% NaCl no black precipitates

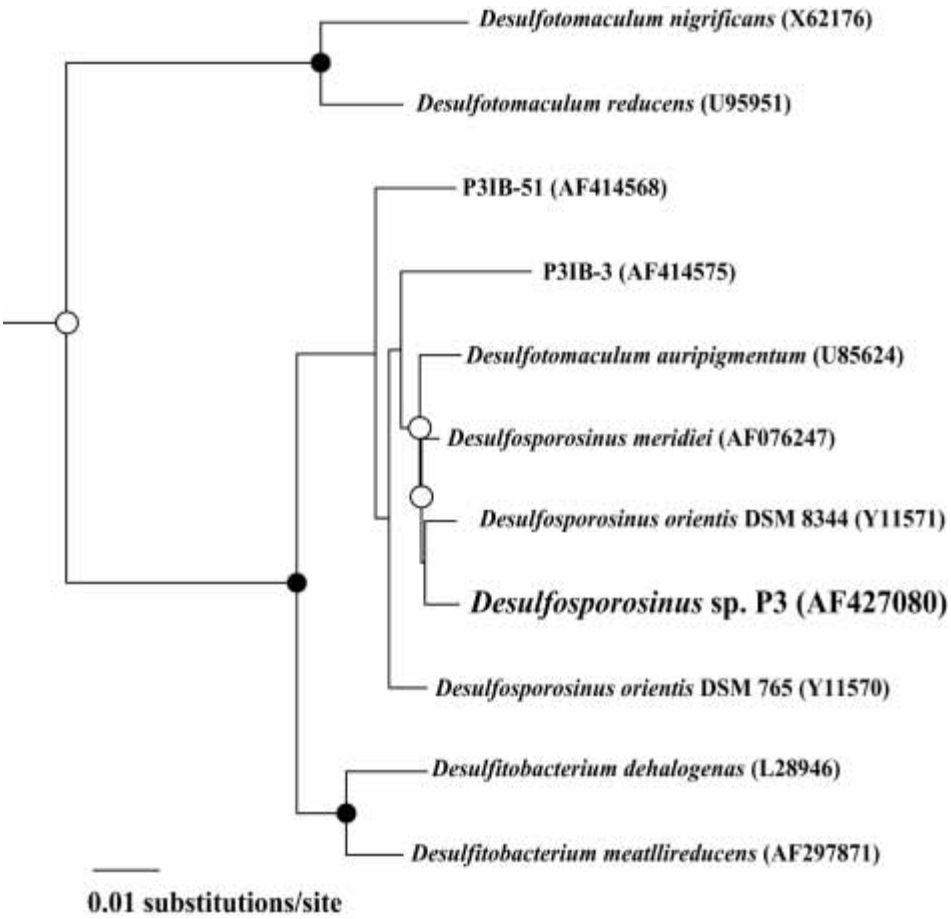

Fig. 1. Evolutionary distance dendogram of an isolate (strain P3) from uranium mine sediment and closely related Gram-positive SRB based on 1220 homologous nucleotide positions of 16S rDNA. *Escherichia coli* was used as the outgroup (not shown). Branch points supported by distance, maximum-likelihood and parsimony estimations (bootstrap values, ($\geq$ 75%) are indicated by solid circles. Marginally supported branch points (supported by most phylogenetic analysis with bootstrap values of 50 to 74%) are indicated by open circles. Branch points without circles are not supported by the majority of analysis. Evolutionary distances are indicated by the sum of horizontal branch lengths. The scale bar represents changes per nucleotide.

of uraninite formed and uranium concentrations did not decrease during incubation (data not shown). These data indicated that strains P3 and DSM 765 did not reduce U(VI) in bicarbonate and salt solutions. As previously reported, U(VI) was reduced by *D. desulfuricans* in the same cell suspensions. Strains P3 and DSM 765 reduced uranium concentrations from 1 mM to > 0.1 mM within 24 h in the cell suspensions without bicarbonate and NaCl (Figs. 2 and 3). Cell suspensions without bicarbonate and NaCl remained at $\sim$ pH 7 throughout the incubation. During incubation, black precipitates accumulated on the bottom of the experimental vessels. There was no decrease in uranium concentrations when cell suspensions of strains P3 and DSM 765 were incubated without lactate or H_2 as electron donors (data not shown). The heat-killed cells and the $CuCl_2$-treated cells of both strains showed no significant decrease in uranium concentrations (Figs. 2 and 3). When 1 mM Na-sulfate was added to the cell suspensions containing either bicarbonate or NaCl, sulfate reduction occurred, as evident from a decrease in sulfate concentrations and sulfide production (data not shown). However, U(VI) was not reduced by the sulfide produced in experiments with either strain.

Cell suspensions of Strain P3 were incubated for 48 h without bicarbonate and 0.85% NaCl. The incubated cells with the black precipitates were observed by TEM. A TEM image of a cell (strain P3) and extracellular U-bearing precipitates is shown in Fig. 4a. EDX analyses showed the precipitates were composed of uranium (data not shown). The analysis of the SAED pattern from the precipitates revealed that the precipitates were identical to uraninite (data not shown). Arrows in Fig. 4a indicate dark bands along the rim of the cell where uranium accumulated.

The oxidation state of uranium in the precipitates characterized by TEM was determined by XANES. Fig. 5 shows

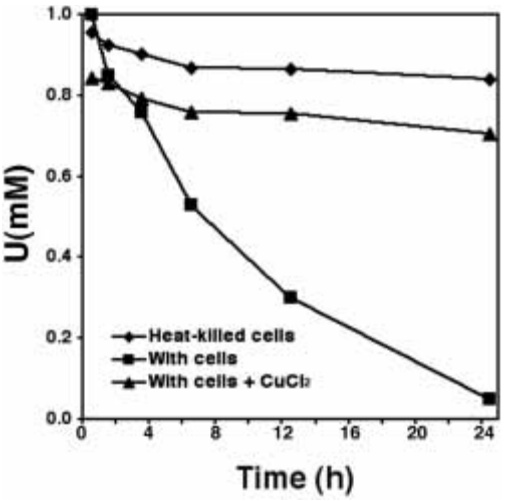

Fig. 2. U(VI) reduction by strain P3 in a pH 7 solution containing 1 mM of Na-lactate and uranyl acetate. The cell protein concentration was at ~ 0.06 mg/ml ($\sim 3.5 \times 10^8$ cells/mL). Each point is the mean of the triplicate measurements and range within less than 10% errors, otherwise indicated.

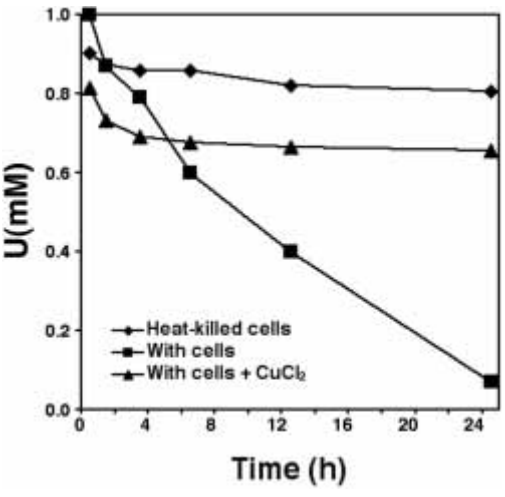

Fig. 3. U(VI) reduction by strain DSM 765 in a pH 7 solution containing 1 mM of Na-lactate and uranyl acetate. The cell protein concentration was at ~ 0.06 mg/mL ($\sim 3.5 \times 10^8$ cells/mL). Each point is the mean of the triplicate experiments. Each point is the mean of the triplicate measurements and range within less than 10% errors, otherwise indicated.

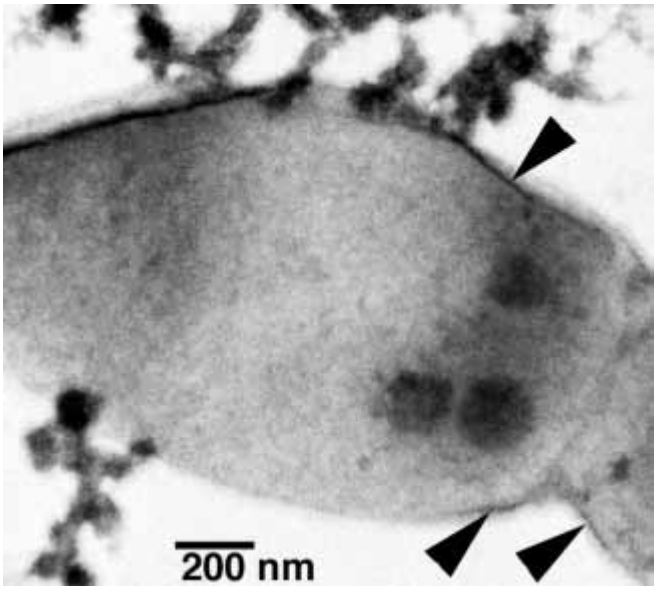

Fig. 4. TEM image showing that the strain P3 cell is associated with extracellular U-bearing precipitates. Arrows indicate the electron-dense line along the rim of the cell.

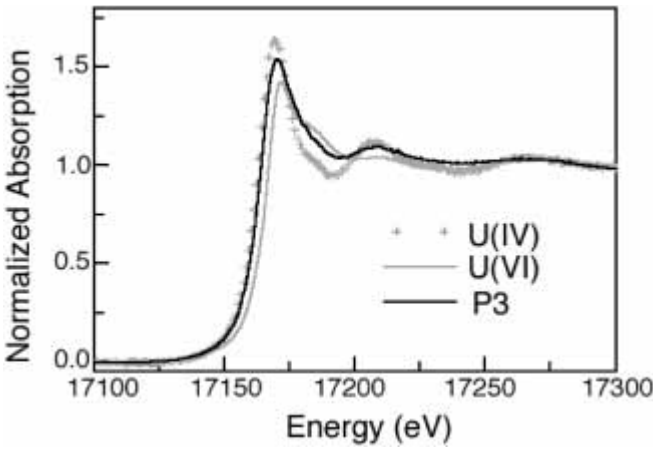

Fig. 5. The averaged normalized uranium L_3-edge absorption data for the strain P3 sample and for the U(IV) and U(VI) standards from UO_2 and UO_3, respectively.

the average of normalized XANES spectra from the strain P3 sample (P3 in Fig. 5), along with the U(IV) and U(VI) standards. The uranium L_3-edge absorption edge from the strain P3 sample is between the U(IV) and U(VI) standards, indicating a mixture of both valence states in the strain P3 sample. By comparing the energy position at half the step height of the strain P3 sample and the U(VI) and U(IV) standards, we estimated $73 \pm 10\%$ U(IV) in the P3 strain sample.

Discussion

The capability of U(VI) reduction by various species of SRB has been tested using five *Desulfovibrio* spp., two *Desulfobacter* spp., one *Desulfobacterium*, *Desulfobulbus*, *Desulfomonile*, and *Desulfotomaculum* sp., under non-growth conditions in the cell suspensions containing bicarbonate, uranyl acetate, and either lactate or H_2 for an electron donor [4]. It was determined that none of the SRB tested, except for all of the *Desulfovibrio* spp., are capable of enzymatic U(VI) reduction [4]. Only two species of SRB, *D. reducens*, and *Desulfovibrio* sp. isolated from a uranium mine, have been reported to be capable of dissimilatory U(VI) reduction [7, 8]. Based on results of laboratory experiments with mixed cultures, we suspected that *Desulfosporosinus* spp. can reduce U(VI). As similar to the previous study [4], neither of strains P3 and DSM 765 reduced U(VI) when the cell suspensions contained bicarbonate. We also tested reduction of U(VI) by the two species in the cell suspensions containing NaCl instead of bicarbonate. However, both of the strains reduced U(VI) in the cell suspensions depleted in bicarbonate and NaCl. As there was no U(VI) reduction when the cell suspensions were incubated with heat-killed cells or when the cell suspensions contained no electron donors such as H_2 and lactate, we infer that both *Desulfosporosinus* spp. catalyze U(VI) reduction coupled to electron-donor oxidation enzymatically rather than nonenzymatic reduction of U(VI) by reducing compounds in the cells or by the electron donors. In the experiments in bicarbonate and NaCl solutions, sulfide was produced by *Desulfosporosinus* sp. strains, yet no uranium reduction occurred. The results indicate that neither sulfide or gram-positive cell surfaces reduce uranium.

Uranium(VI) reduction by both strains was inhibited by addition of 1 mM $CuCl_2$ to the cell suspensions. As 1 mM $CuCl_2$ that inhibits activity of hydrogenase has no effect on sulfate reduction by whole cells of strain DSM 765, but it inhibits sulfate reduction by the lysed cells, it was concluded that strain DSM 765 has hydrogenase in the cytoplasm [19]. As $CuCl_2$ is, however, known to inhibit activities of a broad range of electron transfer enzymes including hydrogenase, we infer that U(VI) reduction by *Desulfosporosinus* spp. was mediated either by single or a subset of electron transfer enzymes. In addition, as reduced uranium minerals were observed in space between the cytoplasmic membrane and cell wall of strain P3, it is suggested that the terminal enzyme donates electrons to U(VI) is located at the outer surface of cytoplasmic membrane or in the space in a soluble form. Triheme cytochrome c_3, which is the terminal enzyme that donates electrons to U(VI) in the case of *Desulfovibrio* vulgaris [20], is absent in strain DSM 765 [21]. Although there was no U(VI) reduction by the hydrogenase of *D. vulgaris* alone [20], Tc(VII) reduction by hydrogenase obtained from *Escherichia coli* and *D. desulfuricans* are possible [22]. It was also reported that Cr(VI) is reduced by a hydrogenase extracted from *Desulfomicrobium norvegicum* and *D. vulgaris* [23]. Hydrogenase or a *c* type cytochrome other than c_3 may be the terminal electron-donating enzyme to U(VI) in *Desulfosporosinus* spp. Reduction of U(VI) by *Desulfosporosinus* spp. was inhibited by 0.25% bicarbonate or 0.85% NaCl in the cell suspensions. These constituents are commonly used to maintain cell integrity and solution pH. Strain DSM 765 was reported to grow using HCO_3^- as the sole electron acceptor with lactate as an electron donor during homoacetogenic fermentation [24]. Thus, *Desulfosporosinus* spp. may selectively reduce HCO_3^- over U(VI). Strain DSM 765 has been reported to grow in a medium with 4% NaCl using sulfate as the electron acceptor. The reason for the effect of 0.85% NaCl on U(VI) reduction is currently unclear.

Desulfosporosinus spp. have been isolated from, or detected by molecular biological techniques at, many subsurface settings enriched with xenobiotic compounds [25–28] or with naturally occurring lignite [29]. *Desulfosporosinus* spp. can reduce perchloroethene (PCE) in pure culture [28], and may play roles in degradation of toluene or phenol in co-culture [27, 30]. As uranium-contaminated sites are often contaminated with toxic organic compounds, stimulation of *Desulfosporosinus* spp. may lead to both degradation of toxic organic compounds and immobilization of uranium from contaminated subsurface groundwater.

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